Application of pomegranate (*Punica granatum* Linn.) fruit extract for accelerating post tooth extraction wound healing

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ABSTRACT

Background: Trauma occurring during tooth extraction can cause complications such as bleeding, infection, fracture and dry socket and constitutes an inflammatory response trigger. Pomegranate (*Punica granatum* Linn.) extract, which contains large amounts of punicalagin and ellagic acid, possesses various qualities, including; anti-inflammatory, anti-bacterial and anti-oxidant. Pomegranate extract can inhibit proinflammatory cytokine production, while also suppressing inflammation response thereby accelerating wound healing. Purpose: This study aimed to analyze the effect of pomegranate extract application to the tooth extraction wounds of *Cavia cobaya* (*C. cobaya*) on the expression of fibroblast growth factor-2 (FGF-2) and transforming growth factor β (TGF-β) on the fourth day of the wound-healing process. Methods: This study used 12 *C. cobaya*, divided into two groups, namely; control and treatment. The subjects were anesthetized, before their lower left central incisor was extracted and the entire socket filled with CMC-Na 3% in members of the control group and pomegranate extract in those of the treatment group. The twelve *C. cobaya* were sacrificed on day 4, their lower jaw subsequently being removed and decalcified for approximately 30 days. The mandibula tissue was stained using an immunohistochemical technique. FGF-2 and TGF-β were used to evaluate the healing process in the extracted tooth socket. Differences in the expression of FGF-2 and TGF-β were evaluated statistically by means of a t-test. Results: This study indicated a significant difference between the control and the treatment groups (p<0.05). The treatment group members whose sockets were filled with pomegranate extract showed high FGF-2 and TGF-β expression. Conclusion: This study confirmed that the administration of pomegranate extract to post-extraction tooth wounds of *C. cobaya* increases the expression of FGF-2 and TGF-β on day 4, thereby accelerating the wound healing process.

Keywords: FGF-2; post extraction wound; Punica granatum Linn; TGF-β

INTRODUCTION

Trauma can occur during tooth extraction resulting in complications such as bleeding, infection, fracture and dry socket.

Periodontal tissue damage caused by tooth extraction also results from inflammation.

The response is triggered by tissue damage in the body and the condition activates macrophages and cytokine synthesis which involves pro-inflammatory activity, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor α (TNFa).

It is essential to control inflammation due to its potential negative effects on the surrounding tissue. The healing process generally consists of three stages, namely; inflammation, proliferation and repair. The control of inflammation will enable the next healing stage to occur.

Research into various types of plants regarded as having medicinal properties beneficial to human health has been widely conducted. One such plant is pomegranate (*Punica granatum* Linn), almost all parts of which have traditionally been used and are believed to promote medicinal activities.

Pomegranate extract possesses anti-inflammatory,
anti-bacterial, anti-oxidant, anti-cancer and anti-fungal properties, with the result that they have been the subject of a considerable volume of research. The phytochemical and pharmacological content of pomegranate, including punicalagin and ellagic acid, can prevent and treat a wide variety of diseases. Ellagic acid promotes anti-inflammatory activity, namely that of reducing IL-6 by inhibiting nuclear factor kappa beta (NF-kB). Pomegranates possess several therapeutic properties applied through a variety of mechanisms and several studies have been conducted into their antioxidant, anticancer and anti-inflammatory roles. It is anticipated that the application of pomegranate extract to tooth extraction sites will accelerate healing due to its anti-bacterial, anti-inflammatory and anti-oxidant properties.

At the healing stage, fibroblast growth factor-2 (FGF-2) and transforming growth factor-β (TGF-β) will stimulate fibroblasts to synthesize type I collagen which is a marker of the healing process. Increased growth factors such as FGF-2, which has the effect of tissue regeneration, play an important role in the wound healing process. TGF-β proteins represent cytokines that control cellular processes, including: cell proliferation, differentiation, adhesion, angiogenesis, apoptosis and immunity maintenance. TGF-β is a multifunctional cytokine which acts as a key regulator of ECM formation and remodeling. Increased TGF-β-1 present in injury sites during the healing process promotes signal tissue regeneration. The purpose of this study was to analyze the stimulating effect of pomegranate extract on Cavia cobaya (C. cobaya) tooth extraction wounds by accelerating the healing process through the expression of FGF-2 and TGF-β on the fourth day. An increase in the expression of FGF-2 and TGF-β was observed.

MATERIALS AND METHODS

This study involved the use of Wistar rats as subjects and received approval from the Ethical Committee of the Faculty of Dental Medicine, Universitas Airlangga, confirmed by ethical clearance certificate number: 53/ HRECC.FODM/5/2015. The material employed consisted of standardized pomegranate extract powder, at a concentration of 2.5% 13, and containing 40% ellagic acid (Xi’an Biof Bio-Technology Co., Ltd China.), while the gel base material was 3% sodium carboxy methyl cellulose (CMC-Na) (Merck). The immunohistochemical staining materials were monoclonal antibodies to FGF-2 (Abcam, ab92337) and TGF-β (Abcam, ab179695).

This study used 12 C. cobaya aged 2.5 months and weighing 150-200 grams as experimental subjects which were acclimatized for a week prior to treatment. The subjects were divided into two groups, namely; the control group (C) administered with 3% CMC-Na gel, and the treatment group (T) whose members received pomegranate extract gel. 3% CMC-Na gel was produced from three grams of CMC-Na powder which was gradually added to 100 ml of water and agitated until homogeneous. 2.5% pomegranate extract gel was produced from 2.5 grams of pomegranate extract powder added to 97.5 grams of 3% CMC-Na gel.

The C. cobaya were anesthetized using a combination of ketamine HCl and diazepam (1:1 cc with a dose of 1 ml/kg bm administered intramuscularly). Extraction of the lower incisors of the subjects was performed using a needle holder and sterile elevator in direct motion (to lingual direction). In the treatment group, the resulting tooth sockets of the subjects were filled with 2.5% pomegranate extract gel, while in the case of the control group 0.1 ml of 3% CMC-Na gel was inserted into each socket with an insulin syringe. Extraction wounds were sutured using non-absorbable threads. The control and treatment group subjects were sacrificed on the fourth day post-treatment, the mandible being extracted following removal of the mandibular angles.

The mandibular extracted from the subjects was soaked in buffer formalin for 24 hours, subsequently decalcified with 10% ethylene diamine tetra acetic acid (EDTA) for approximately 30 days to soften the mandibular bone tissue, thereby enabling it to be dissected into small, rectangular pieces. Embedding was performed by means of paraffin solution at a temperature of 56-59 ºC. A paraffin block was cut to a thickness of 5 μm before immunohistochemical tissue staining was conducted by using monoclonal anti FGF-2 antibodies to observe FGF-2 expression cells and anti TGF-β monoclonal antibodies to observe TGF-β expression cells. Observations with light microscopes and cameras (Olympus) were carried out at 400X magnification. The expression of FGF-2 and TGF-β was measured by counting the number of cells (macrophages) expressing FGF-2 and TGF-β.

Data obtained from the results of the study were analyzed using a t-test to determine the differences in expression of FGF-2 and TGF-β between the control and treatment groups with a significance of 0.05.

RESULTS

FGF-2 expression cells in the control group (CMC-Na) and the treatment group (pomegranate extract) are shown in Figure 1, while TGF-β expression cells in the control group (CMC-Na) and treatment group (pomegranate extract) can be seen in Figure 2. The mean and standard deviation of FGF-2 and TGF-β expression is presented in Table 1. The difference between control and treatment groups can be seen in Table 2.

The t-test for the expression of FGF-2 and TGF-β demonstrated significant differences (p<0.05) between the control and the treatment groups.
Figure 1. Post-extraction FGF-2 expression on day 4 after. A) in the control group; B) in the treatment group (400x magnification, arrow indicates immunoreactive cells, highlighted in brown).

Figure 2. Post-extraction TGF-β expression on day 4. A) in the control group; B) in the treatment group (400x magnification, arrow indicates immunoreactive cells, highlighted in brown).

Figure 3. FGF-2 and TGF-β expression of control and treatment groups.

Table 1. The mean and standard deviation of FGF-2 and TGF-β expression in the control and treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>FGF-2</th>
<th></th>
<th>TGF-β</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
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<td>6</td>
<td>4.83</td>
<td>1.941</td>
<td>7.67</td>
<td>1.211</td>
</tr>
<tr>
<td>Treatment</td>
<td>6</td>
<td>12.17</td>
<td>2.137</td>
<td>10.83</td>
<td>2.994</td>
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</tbody>
</table>

Table 2. T-test of FGF-2 and TGF-β expression in the control and treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Expression</th>
<th>T value</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - Treatment</td>
<td>FGF-2</td>
<td>6.223</td>
<td>0.000</td>
</tr>
<tr>
<td>Control - Treatment</td>
<td>TGF-β</td>
<td>2.401</td>
<td>0.037</td>
</tr>
</tbody>
</table>
DISCUSSION

NF-kB is a key transcription factor for macrophages and required for the induction of a number of inflammatory genes, including those that encode TNF-α, IL-1β, IL-6 and cyclooxygenase-2. Trauma will cause NF-kB translocation to the cell nucleus, an event triggering the transcription process for the formation of various pro-inflammatory cytokines. The release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and cyclooxygenase-2 induces changes in the tissue referred to as inflammation. The application of pomegranate extract high in ellagic acid to post-tooth extraction wounds can inhibit NF-kB activity. Such inhibition of NF-kB activation can occur by means of three mechanisms, namely; blocking the incoming stimulating signal (e.g. binding ligand to the receptor) resulting in an imperfect signal effect; interfering with the cytoplasm stage of the NF-kB activation pathway by inhibiting activation of IkB kinase (IKK) or IkB (inhibitory protein kappa B) degradation; and, lastly, inhibiting nuclear NF-kB activity which inhibits translocation into the nucleus and hinders NF-kB-DNA binding.

The obstacle of NF-kB translocation into the nucleus causes inflammation to be appropriately regulated in order that the production of FGF-2 and TGF-β by macrophages is increased. It has been proven in this study that an increase in FGF-2 and TGF-β expression occurs in post-extraction wounds with the result that healing is accelerated because FGF-2 and TGF-β stimulate the formation of granulation tissue. In addition to FGF-2 and TGF-β, macrophages secrete vascular endothelial growth factor (VEGF) which modulates endothelial cell proliferation and causes angiogenesis.

Together with IL-10, TGF-β is a strong anti-inflammatory cytokine that actively reduces pro-inflammatory TNFα, IL-1β and IL-2 among other proteins. TGF-β also plays an important role in tissue regeneration, cell proliferation and cell differentiation. While FGF-2 is an effective growth factor indicator with the potential to affect tissue repair and regeneration.

FGF-2 is mainly produced by macrophages on day 2, while active fibroblasts proliferate from day 3. TGF-β was released immediately after the trauma in the inflammatory phase and increased in the proliferation phase of the day 3. Therefore, studies of FGF-2 and TGF-β in post-extraction wounds were carried out on the fourth day after treatment.

Analysis of the results of this study confirmed that the number of cells expressing FGF-2 and TGF-β was higher in post-extraction wounds after the administering of standardized pomegranate extract containing 40% ellagic acid compared to the control group. This is because ellagic acid possesses anti-inflammatory qualities which reduce inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), TNF-α and IL-6 by suppressing NF-kB activation, thereby enabling the effective control of inflammation in cavia cobaya. This, in turn, not only leads to a decrease in the transcription process of pro-inflammatory cytokines, but also activate genes that produce anti-inflammatory cytokines, namely: IL-10, FGF and TGFβ. IL-10 inhibits pro-inflammatory cytokines, while FGF stimulates fibroblast cell proliferation. As a result of this stimulation, fibroblast cells secrete TGFβ-1 which affects fibroblast cell proliferation in an autocrine manner and causes increased collagen synthesis which is indicative of a healing process.

This study is supported by other research findings confirming that the ellagic acid content of pomegranate fruit extract can also reduce proinflammatory cytokine activity in the sockets of diabetic mice.

FGF-2 is mitogenic for several cell types found in wound sites, including fibroblasts, which contribute significantly to the wound healing response. This hypothesis has been corroborated by other research indicating that the application of local FGF-2 stimulates tissue repair. Previous studies revealed that, after tooth extraction, FGF-2 decreased in untreated diabetic rats compared to those treated with ellagic acid and that a correlation existed between the decrease in FGF-2 expression and wound healing disorder. FGF-2 expression decreased during wound healing in healing-impaired genetically diabetic subjects compared to control group subjects. The expression of FGF-2 was found to be present in both undamaged and wounded skin, while it increased after the skin had been lacerated. In this study, FGF-2 was identified as a wound-regulated protein. The expression of FGF-2 was found to increase after injury in healthy subjects, but not in diabetic ones. In conclusion, the administration of pomegranate extract to post-extraction wounds of C. cobaya increased the expression of FGF-2 and TGF-β on day 4 and accelerated the wound-healing process.

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DOI: 10.20473/djmkv51.i4.p189–193